Valence of physical stimuli, not housing conditions, affects behaviour and frontal cortical brain activity in sheep

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Abstract: Modulation of short-term emotions by long-term mood is little understood but relevant to understand the affective system and of importance in respect to animal welfare: a negative mood might taint experiences, whilst a positive mood might alleviate single negative events. To induce different mood states in sheep housing conditions were varied. Fourteen ewes were group-housed in an unpredictable, stimulus-poor and 15 ewes in a predictable, stimulus-rich environment. Sheep were tested individually for mood in a behavioural cognitive bias paradigm. Also, their reactions to three physical stimuli thought to differ in their perceived valence were observed (negative: pricking, intermediate: slight pressure, positive: kneading). General behaviour, activity, ear movements and positions, and haemodynamic changes in the cortical brain were recorded during stimulations. Generalised mixed-effects models and model probabilities based on the BIC (Bayesian information criterion) were used. Only weak evidence for mood difference was found. Sheep from the unpredictable, stimulus-poor housing condition had a somewhat more negative cognitive bias, showed slightly more aversive behaviour, were slightly more active and moved their ears somewhat more. Sheep most clearly differentiated the negative from the intermediate and positive stimulus in that they exhibited more aversive behaviour, less nibbling, were more active, showed more ear movements, more forward ear postures, fewer backward ear postures, and a stronger decrease in deoxyhaemoglobin when subjected to the negative stimulus. In conclusion, sheep reacted towards stimuli according to their presumed valence but their mood was not strongly influenced by housing conditions. Therefore, behavioural reactions and cortical brain activity towards the stimuli were hardly modulated by housing conditions.

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Valence of physical stimuli, not housing conditions, affects behaviour and frontal cortical brain activity in sheep

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Modulation of short-term emotions by long-term mood is little understood but relevant to understand the affective system and of importance in respect to animal welfare: a negative mood might taint experiences, whilst a positive mood might alleviate single negative events.

To induce different mood states in sheep housing conditions were varied. Fourteen ewes were group-housed in an unpredictable, stimulus-poor and 15 ewes in a predictable, stimulus-rich environment. Sheep were tested individually for mood in a behavioural cognitive bias paradigm. Also, their reactions to three physical stimuli thought to differ in their perceived valence were observed (negative: pricking, intermediate: slight pressure, positive: kneading). General behaviour, activity, ear movements and positions, and haemodynamic changes in the cortical brain were recorded during stimulations. Generalised mixed-effects models and model probabilities based on the BIC (Bayesian information criterion) were used.

Only weak evidence for mood difference was found. Sheep from the unpredictable, stimulus-poor housing condition had a somewhat more negative cognitive bias, showed slightly more aversive behaviour, were slightly more active and moved their ears somewhat more. Sheep most clearly differentiated the negative from the intermediate and positive stimulus in that they exhibited more aversive behaviour, less nibbling, were more active, showed more ear movements, more forward ear postures, fewer backward ear postures, and a stronger decrease in deoxyhaemoglobin when subjected to the negative stimulus.

In conclusion, sheep reacted towards stimuli according to their presumed valence but their mood was not strongly influenced by housing conditions. Therefore behavioural reactions and cortical brain activity towards the stimuli were hardly modulated by housing conditions.

Keywords: mood, emotion, cognitive bias, functional near-infrared spectroscopy (fNIRS), ear movements.

1 Introduction

Repeatedly experiencing negative or positive events is thought to influence the general mood of humans and animals [1], i.e. the long-term emotional state. Mood in itself is then thought to feed back into short-term emotional reactions. Knowledge of the interplay of mood and emotional reactions is important for understanding how the affective system functions, and is of practical relevance in terms of animal welfare. It has typically been assumed that negative mood would taint all emotional reactions in humans (see depression research [2,3]). Non-clinical negative mood may have a different effect, however, at least in animals [4] if not in humans [5]. We have found that sheep in a more positive mood reacted less strongly towards a negative as well as towards a positive stimulus in respect to their behaviour, physiology [4], and frontal cortical brain activity [6]. A first aim of our study was therefore to differentiate between the two hypothesised effects of negative mood, i.e. whether emotional reactions of our study species, the sheep, would be generally shifted towards the negative when the animals are kept in housing conditions likely to induce a negative mood or whether these animals would react more negatively towards negative as well as more positively towards positive stimuli.

In animals, finding indicators for emotions is notoriously difficult [7,8,9]. This is even more so, since animal welfare research has shifted from focusing purely on negative conditions (e.g. [10, 11]) to include conditions thought to elicit positive reactions in animals (e.g. [12]). Given this wider view, the question has arisen as to whether activity in the brain, physiological, and behavioural reactions of animals encode for the valence of a situation. Valence is the emotional value attributed to a situation or stimulus on an axis that ranges between a pole of negativity to a pole of positivity (e.g. [1,13]; in humans e.g. [14,15]). In previous studies, we found evidence for such encoding of valence. When moving from negative to positive situations, this encoding consisted of a decrease in the number of ear movements, in the proportion of forward ear postures, in the proportion of asymmetric ear postures in sheep (reflecting changes in behaviour; [4,16,17]), a decrease in heartbeat and breathing rate in

86 sheep (changes in physiology; [4,17,18]), and weaker general frontal cortex activation,
87 associated with a shift of the activation to the left side of the brain, in goats (changes in brain
88 activity; [19]). Similar results were found in other studies in relation to ear postures in sheep
89 [20, 21], pigs [22], and facial expressions in mice [23]. The brain activation, behavioural, and
90 physiological changes observed in our own and other's studies with regard to stimulus
91 valence can therefore be viewed as indicators of the animals' short-term emotional reactions.
92 This leads to the second aim of our study, in that we wanted to further validate the use of
93 cortical brain activity and behaviour as indicators for valence, i.e. to investigate whether
94 consistent changes in these indicators occur when the valence of a stimulus is changed.

95 The interpretation of changes in indicator variables reflecting brain activity, behaviour, and
96 physiology has often been difficult to date because experimental situations were used that
97 differed not only in terms of their valence, but also in terms of e.g. the degree of arousal they
98 elicited (e.g. [24], in humans). Previous research may thus have been limited in that a
99 number of the effects attributed to the valence of a situation might actually have been caused
100 by other aspects of the situation, such as arousal. Here, we present an experiment in which
101 stimulus valence was varied across a set of three stimuli while other aspects of the stimuli
102 were kept as similar as possible. This had the effect that arousal remained as similar as
103 possible. We subjected sheep to three physical stimuli which were all applied on the upper
104 thorax of the animals by a mechanical stimulus device. These stimuli ranged from a pricking
105 stimulus, to light pressure, to gentle kneading, with a view to eliciting a negative,
106 intermediate, and positive behavioural reaction, respectively, as well as differences in brain
107 activity. Specifically, we observed general behaviour throughout the stimulation sessions. In
108 addition, for each individual stimulus, we automatically tracked ear movements and positions
109 [16,17,25] and traced brain activity in the frontal cortex [26,27] using functional near-infrared
110 spectroscopy (fNIRS; [6,19]). This method is of specific interest here because it allows for
111 non-invasive measurement of cortical brain activity on freely moving animals.

The sheep subjected to the different physical stimuli originated from two groups. One of the groups was kept in an unpredictable, stimulus-poor housing environment, and the other in a predictable, stimulus-rich housing environment [28,29] to induce different mood states. This allowed us to address the first aim of our study and investigate how mood modulates the effects of our physical stimuli. In addition, we wanted to have an independent assessment of mood by conducting a cognitive bias test (e.g. [7,13,30]). In this test, animals are trained to expect a reward related to a specific cue and a punishment related to another cue (e.g. different sound frequencies). Their reaction is then tested in response to an ambiguous cue (an intermediate frequency). If animals react to the intermediate cue similar to the cue related with the punisher, their reaction is considered to be pessimistic. We expected the sheep from the unpredictable stimulus-poor ("unpredictable poor") housing environment to show a more pessimistic reaction in the cognitive bias test than the sheep from the predictable stimulus-rich housing ("predictable rich").

2 Materials and methods: general procedure

This study consisted of three main parts: (1) mood induction by housing condition (predictable rich versus unpredictable poor), (2) assessment of the induced mood by using a cognitive bias test [7,13,30], and (3) impact of three valences (negative, intermediate, positive) of physical stimuli on cortical brain activation and behavioural reactions. The three parts were in fact conducted in the sequence 1-3-2 in order to capture any effect of mood induction in part 3 as effectively as possible. Mood induction was continued throughout parts 3 and 2 and sheep were left undisturbed in their respective housing conditions for two weeks between the different parts to allow mood to stabilise. As part 2 is a prerequisite for the interpretation of part 3 we will nevertheless present part 2 before part 3.

137 *2.1 Animals*

138 Thirty-one Lacaune ewes were purchased in two lots (February and April 2011) from two
139 different farms and housed as one group in an open-front pen (58 m^2) at Agroscope in
140 Tänikon. One lamb died for unknown reasons a few days after being brought to the
141 Research Station, and another sheep was put down after breaking its leg in the home pen
142 (both incidents were unrelated to the experimental set-up). For the actual measurements,
143 twenty-four non-gestating, non-lactating sheep (12 per housing group) were randomly
144 chosen. The other five sheep enlarged the housing groups but were not tested.

145

146 *2.2 Ethical note*

147 This project was assessed by the Swiss National Science Foundation, and the necessary
148 authorisation was granted by the cantonal authorities (Canton of Thurgau permit nos. F6/10
149 and F4/11 for the conducting of animal experiments), ensuring that no undue impairment of
150 animal welfare was caused by the experiment.

151

152 *2.3 Mood induction*

153 The 29 animals (aged 5.6 ± 0.8 months) were assigned to two groups for mood induction
154 (from July 2011 onwards), balanced for farm of origin and purchase lot: Predictable rich (n=
155 15) and unpredictable poor (n= 14),

156 The sheep from the predictable rich group had free access to water and were fed twice a day
157 (7.30-8.00 am and 4.30-5.00 pm) a ration of hay such that the hay rack was empty before
158 being re-filled. Animals were exposed to natural daylight and temperatures. The open-front
159 pen (58 m^2), was divided into a feeding area (16 m^2; concrete floor; 6.75 m hayrack) and a
160 lying area (42 m^2; deep litter, 4.0 m hayrack) structured by lying niches. An exercise yard
161 (20.8 m^2; concrete) and pasture were also provided. Weather permitting (i.e. except during

heavy rainfall or snow), the exercise yard was accessible from between 9.00-10.00 am to between 5.00-7.00 pm, whilst access to the pasture was provided for 2 to 7 hours per workday.

For inducing a more negative mood the access to food and water was made unpredictable by an automatic system since such irregular times of food distribution induced frustration in cows [31]. A shutter locking the 7.6-m-long hayrack was closed directly before feed provisioning (7.30-8.00 am and 4.30-5.00 pm) and automatically opened after a random interval of a maximum of two hours. Our sheep would typically stand up immediately when the shutter was closed, approach the shutter and start clawing at the closed shutter. Water was available twice daily at unpredictable times. Additionally, the light cycle was irregular in that light (artificial light or natural daylight) was made available for 6 to 16 hours starting at a time point between 4.00 am and 11.00 pm. Such irregular lighting is known to influence emotional state [32,33]. The pen consisted of an unstructured deep-litter straw area (22.4 m^2) with closed walls that denied the animals visual stimulation from outside the pen.

2.4 Assessing mood: cognitive bias test

We assessed mood from mid-May to July 2012 and used a spatial variant of the cognitive bias test in which sheep were trained to expect a reward in one location and a punishment in another location, and ambiguous stimuli were presented in between. One box at any one time presented in these locations served as cue. The test took place in an arena built with wire-mesh fences (height: 1.90 m) and measuring 3.6 m x 4.3 m, with a 2-m-deep waiting area along the shorter side of the arena. To prevent sheep being distracted, the fences were covered with bales of straw and sheets on their exterior. Because sheep were generally hesitant in crossing the border between the waiting area and the testing arena they were always prompted acoustically by saying ‘go’ in a neutral tone to enter the testing arena after opening the door between the two areas. Boxes were placed one at a time along the shorter

188 side of the arena facing the waiting area. Boxes were closed at the start of each trial, and
189 opened automatically if sheep came closer than a distance of 1.1 m. The opening of a box's
190 lid was triggered by a motion detector (EPM360, ELBRO AG Swiss Technology Company)
191 placed 1.51 m above the box in a tube (detector located 29.5 cm below top of the tube; total
192 tube length 51 cm, Ø 13 cm).

193 The 12 focal sheep from each housing group were divided into four sub-groups of three
194 sheep. Two sub-groups from each housing condition were trained in the morning and the
195 other two were trained in the afternoon. About half of the sheep in both housing groups had
196 participated in a pilot cognitive bias test in February 2011, i.e. roughly one year before the
197 current test. Sheep were trained and tested in several steps, as follows:

198 Training step 1: Sheep were habituated twice to the testing arena in their respective sub-
199 group of three.

200 Training step 2: Each sub-group was confronted twice with a box containing food ("positively
201 reinforced box") positioned in the middle in order to familiarise them with the food reward (a
202 mixture of UFA 114 Kombi Pro Rumin, Zollikofen, Switzerland; sugarbeet slices, Cornflakes
203 and rolled oats). On the first occasion, the box was open to allow the sheep to see the food
204 inside, whilst from the second trial onwards the box was initially closed, opening only when at
205 least one of the sheep approached the box in question.

206 Training step 3: Sheep were individually trained to go up to the box and eat from it. To avoid
207 a location bias, the box was positioned in the right-hand corner of the testing arena for half of
208 the sheep of each housing condition and in the left-hand corner for the other half. Each
209 sheep was always trained at the same time of day (balanced for the two housing conditions)
210 and went through a maximum of one session per day, with each session consisting of five
211 individual trials. In the first trial of this step, sheep were led to the box until it opened if they
212 did not approach voluntarily. In each trial from this step onwards, sheep were allowed to
213 leave the testing arena when they turned towards and approached the door of the waiting

214 area. Each sheep underwent at least seven training sessions. If sheep promptly approached
215 the positively reinforced position in at least three trials in three successive sessions, they
216 moved to the next step.

217 Training step 4: Sheep were also confronted with a box positioned in the opposite corner to
218 the positively reinforced box. From this box ("negatively reinforced box"), a cloth figure
219 (height: 89 cm) was raised by a blower situated directly behind the box but outside the
220 testing arena (Performer StromCube, MailShop GmbH Augenoptik, Mühlacker, Germany).
221 To refrain from giving the animals an auditory cue with respect to box type, the blower was
222 left on throughout all of the trials regardless of the position of the box. The blower was
223 always situated behind the location of the negatively reinforced box for a given sheep and
224 might thus have strengthened the reaction towards this location. When confronted with the
225 stimulus of the negatively reinforced box, sheep consistently demonstrated withdrawal
226 behaviour. In the first trial of this step sheep were also led to the box until it opened if they
227 did not approach it voluntarily. The five trials in each session of this step consisted of three
228 trials with the positively reinforced position and two with the negatively reinforced position.
229 The training sessions always ended with the positive position, and no position, whether
230 positive or negative, occurred more than twice in a row. Apart from this, the sequences were
231 set up at random. Sheep went into the experimental trials when they readily approached the
232 positively reinforced position and refrained from approaching the negatively reinforced
233 position in three successive sessions (i.e. 15 "correct" trials in a row).

234 Experiment: Sheep underwent three sessions of five trials each, with one session per day on
235 three successive days. Each session included one trial with an ambiguous position at either
236 30%, 50%, or 70% of the distance between the positions of the negatively and positively
237 reinforced boxes. The ambiguous boxes were left empty, whilst the other boxes worked as
238 during the training sessions. In all sessions of the experiment, the sequence of the five trials
239 was as follows: negatively, positively, ambiguous, negatively, and positively reinforced
240 position. The sequence of these trials was kept constant in order to keep sequential effects

241 constant. Thus, the influence of the negatively reinforced position with respect to the reaction
242 to the ambiguous position was minimised, and all sessions ended with the positively
243 reinforced position. All six possible sequences of the three ambiguous positions across the
244 three sessions were randomly assigned to two sheep of each housing condition. The main
245 outcome recorded in each trial was whether the sheep opened the box ('go response') or not
246 ('no-go response').

247

248 *2.5 Cortical brain activity and behavioural reactions in response to stimulus valence*

249 *2.5*.1 General Procedure

250 To induce emotional reactions, the same 24 focal sheep used in the cognitive bias test were
251 confronted with three stimuli thought to differ in valence (presumed negative, intermediate,
252 and positive; February to April 2012). All stimuli were of the same sensory modality (touch),
253 and were therefore expected to vary as little as possible in terms of the elicited arousal. To
254 enable the stimuli to be applied consistently and to reduce the influence of a human
255 experimenter as far as possible, the three stimuli were applied by mechanical devices, and
256 included pricking (presumed negative), slight pressure (intermediate) and kneading (positive;
257 see 2.5.2).

258 Because we were not interested in reactions to the novelty of the stimuli, sheep were
259 habituated to all the stimuli before testing, as described below. We also assumed that sheep
260 would differentially habituate to the different stimuli. We therefore used the fewest habituation
261 trials, namely two, for slight pressure because we assumed that this weak stimulus did not
262 need many trials to be familiarised with. We used more trials for pricking, namely five,
263 because we wanted that the animals knew the stimulus without complete habituation. Even
264 more trials were used for kneading, namely 10, because we had observed in a previous
265 experiments that sheep only started to compete for being groomed seemingly appreciating
266 the physical sensation after several trials.

Habituation step 1: Focal sheep were randomly assigned to groups of three, and were allowed to explore the test pen (2.54 m x 1.97 m, in the corner of a building with 1.21-m-high wooden walls on the two remaining sides) once as a group. While in the test pen as a group, each sheep was fitted with the harness of the stimulus device once for approx. 5 min without activation of the device.

Habituation step 2: Sheep were led individually and once daily at most to the test pen. They underwent 15 habituation sessions within 3 weeks, had a 4-week pause due to construction work close to the housing and testing pens, and were re-habituated to the stimuli in 2 sessions (17 sessions in total). The actual experiment was conducted one month after construction work. In this time the sheep could re-habituate if necessary to the respective housing conditions. In the 17 sessions of habituation step 2, sheep were first habituated to the active stimulus device over eight sessions. For the remaining nine sessions, the head collar for the functional near-infrared spectroscopy (fNIRS) sensor was worn additionally. In each of these two habituation phases, stimulus length was increased from 5 to 10 min. Sheep were confronted with one type of stimulus per session, alternating between the types. The full sequence of the 17 habituation sessions was (without fNIRS sensor): P5, P5, P10, P10, I5, N5, P5, N10; (with fNIRS sensor): P5, I5, P5, N5, P10, N10, P10; (after interruption): N10, P10 (the letter indicating the type of stimulus, Negative, Intermediate, Positive and the number the stimulus duration in minutes).

Experiment: Sheep were confronted with each of the stimuli in the experimental pen at the same time of day on three consecutive days, one session with one type of stimulus per day. All six possible sequences of the three stimuli were randomly assigned to two sheep per housing group. On each day of the experiment, six sheep were tested, three sheep from one housing condition in the morning and three from the other in the afternoon (alternating morning and afternoon between the two housing conditions). The experimental sessions of all animals were finished within three weeks. To calm the experimental sheep, which helped to achieve more reliable fNIRS measurements, they were always accompanied by the same

familiar experimenter in the experimental pen. The experimenter did not, however, interact with the sheep.

2.5.2 Stimulus devices

Each experimental session started with a five-minute acclimatisation period, followed by a 30-sec pre-stimulus period. Stimulus presentations lasted for 45 sec and were repeated 12 times. To avoid neuronal and behavioural reactions owing to expectations, intervals between the stimuli were varied randomly by between 55 and 65 sec. A 30-sec post-stimulus period followed the final stimulus.

Fixed onto a harness, the mechanical stimulus device was positioned at the front of the animal's trunk (between its front legs and neck). Intense localised pressure (pricking), which was chosen as a negative stimulus, was created by fixing one dull metal pin (3.5 cm) in each corner of a metal plate (4.9 x 7.1 cm). These pins closed towards the centre of the plate, pinching the sheep's breast likely to induce slight pain without injuring the skin (one pinching event lasted 15 sec). The intermediate stimulus consisted of the application of a smooth metal plate (5.5 x 6.5 cm) with slight pressure to the sheep's breast thought to induce a physical stimulation that was neither specifically positive nor negative. Kneading, which was meant to simulate grooming by a human experimenter (see [4,17]), was used as a positive stimulus, and was performed by the same metal plate used for the intermediate stimulus, to which four wooden hemispheres (diameter 2.3 cm) were attached. This device was moved up and down the neck rhythmically (1.2 s per motion sequence) within a radius of approx. 3.6 cm. Similar to the observations of [17] at least half of our sheep were found to be willing to compete for being groomed by a human showing that they perceived grooming, in principle, as rewarding (qualitative observations conducted after the end of this experiment).

Whereas the positive and intermediate stimuli were based on the same mechanical device, the negative stimulus had to be fixed onto a different but virtually identical harness. The computer program DasyLab 4.0 controlled the stimuli, as well as logging actions of the

device. A computer was connected via USB with a Personal Data Acquisition System 56 (PDAQ56; IOtech, Measurement Computing Corporation, Norton). This formed the interface between the software and hardware, and sent the signals to a PIC processor (18F1320; Microchip, Arizona). Based on the digital input signal, the PIC processor produced a pulse-position modulation signal (PPM signal) which was compatible with a transmitter (Jeti Duplex 2.4 GHz; Jeti model, Příbor). The transmitter wirelessly transferred the data to the receiver (Jeti Duplex R5; Jeti model, Příbor) on the sheep, which then converted the received data into servo signals. Four servo motors (MKS, DS 450; MKS Yilan City) for each metal plate were ultimately responsible for the movement of the metal plate or the pins, respectively.

2.5.3 Cortical brain activity: measurement of haemodynamic processes

Neuronal activity during stimulation is reflected in changes in cortical oxygenation. These changes in oxy- and deoxyhaemoglobin concentrations ([O_2Hb] and [HHb]) in the cortical area can be measured by near-infrared spectroscopy (fNIRS; [6,34]). Brain activation is usually thought to be reflected by a concurrent increase in [O_2Hb] and decrease in [HHb] [34]. Changes in [O_2Hb] and [HHb] were observed using a wireless sensor (7 cm x 4.5 cm) with two detectors and four different light sources of two wavelengths each (16 light paths in total; LED at 760 and 870 nm peak emission wavelength; source–detector distances of 14 and 22 mm; [35]). The sensor was fixed on the head of the sheep towards the front of the skull so as to cover the brain as close to the frontal cortex as possible. The sheep's head was (re-)depilated every evening before measurements were taken, in order to minimise hair artefacts in the fNIRS measurements.

The signal was digitised with a sampling rate of 100 Hz. Data were transmitted wirelessly to a host computer for storage and subsequent processing. Data were filtered for eight paths (all possible combinations of right/left, caudal/cranial, shallow/deep), resulting in values at 1 Hz. Absolute [O_2Hb] and [HHb] changes in comparison to the pre-stimulus interval (which was set to zero) throughout each stimulus were calculated on the basis of the raw attenuation data, i.e. the strength of light that was picked up by the detectors [36]. To reduce

carry-over effects from one stimulus to the next, we only included a 15-sec pre-stimulus, 45-sec stimulus and 15-sec post-stimulus duration in our analysis.

2.5.4 Behavioural measurements

Throughout all of the experimental sessions, the following behaviours were counted: (1) aversive behaviour patterns (e.g. [20]), including shaking (head or whole body), stamping (jerky movement of fore or hind leg towards floor), bucking (jumping with hind legs or all four legs), and moving backwards (at least one step with front or hind legs not followed by bucking or shaking); (2) vocalisations (e.g. [37]); (3) rearing up the walls (forelegs on the sidewalls of the pen or on the experimenter), indicating a motivation for vigilance; (4) nibbling (manipulating the clothes of the experimenter with the mouth; re-counted after a 10-sec pause without nibbling; as soon as a sheep started to nibble the experimenter, its muzzle was forced away with a slow movement of the experimenter's arm), indicating a motivation for social contact. Each of these behaviour patterns was directly observed by one of two experimenters and counted by tally counters (HC-2, Voltcraft, Germany). Each experimenter always observed the same behaviour patterns.

Ear postures and movements have previously been shown to be potential indicators of emotional reactions in sheep (e.g. [16,17]). In order to save work effort compared to video observations and to increase the accuracy of such ear-movement data, we used a system for automatically tracking movements (Trackpack4, Advanced Realtime Tracking GmbH, Weilheim, Germany). This system consisted of four infrared- sensitive cameras fitted above the test pen. Small reflective marker balls (Ø 16 mm, weight 2.6 g) were located by the system in 3D at 6 Hz. We used one head target (so-called 6D target) on the centre of the sheep's head and two ear targets (so-called 3D targets) attached to the back of the sheep's ears, making use of eartags in both ears of the sheep. Whereas the ear targets consisted of simple balls and could thus be located in 3D with respect to their absolute location, the head target consisted of a specific configuration of a total of four reflective marker balls (142 g). In addition to being located in absolute 3D space, the orientation of the head target (roll, pitch,

374 yawn angles) could be estimated. Based on this information, we calculated the relative
375 position of the ear targets to the head target in the form of horizontal and vertical angles that
376 described how far forward (or back) and up (or down) the ears were positioned in relation to
377 the point in the centre between the sheep's ears (estimated to be 7 cm lower than and 3.5
378 cm behind the head target).

379 For each stimulation (of 45 sec) and its respective pre- and post-stimulus phases (each
380 lasting 15 sec), we then calculated each sheep's general activity (cumulative distance
381 covered by the head target divided by length of phase), the amount of ear movements (sum
382 of the absolute differences between successive horizontal angles of both ears divided by
383 length of phase), the proportion of time when both ears were pointed forwards (forward ears;
384 both ears pointed more than 0 horizontal degrees forwards), the proportion of time when both
385 ears were in a backwards position (backward ears; both ears pointed more than 10
386 horizontal degrees backwards), the proportion of time ears were relaxed (passive ears;
387 vertical angle more than 30 degrees below the horizontal), and the proportion of time that the
388 left ear was positioned more to the front than the right ear (left-asymmetric ears; left ear
389 positioned more than 5 horizontal degrees more forwards than right ear per all ear positions
390 with more than 5 degrees difference in their horizontal angle). No data were logged in 5% of
391 the phases, whilst over 50% and 80% of the sequential data were logged in 77% and 57% of
392 the phases, respectively.

393

394 *2.6 Statistics*

395 Mood induction was simultaneously performed on all sheep of a given group, using exactly
396 the same manipulations of feed, water and light on all animals in the group. It could therefore
397 be argued that in effect our sample size is only two. Despite this, the individually tested
398 sheep were used as the unit of replication in all of our statistical evaluations. It seems highly
399 unlikely that sheep in the housing groups could have affected each other in a way that was

400 unrelated to the long-term affective state of the animals and that would nevertheless have
401 consistently affected the response in our individual tests. If sheep had affected each other by
402 e.g. emotional contagion, this would only strengthen our experimental mood induction.

403 We modelled our data with generalised linear mixed-effects models [38] in R version 2.14.1
404 and 2.15.1 [39] using package lme4 [40]. Assumptions, i.e. homoscedasticity of errors,
405 normality of errors and random effects, were examined using graphical analysis of residuals.
406 We selected statistical models for presentation based on their ranking according to the model
407 weights derived from the Bayesian information criterion (BIC). The models chosen by the BIC
408 were simpler than those chosen by the more-classic Akaike information criterion (AIC; [19]).
409 Because we were interested in the causal relationship between predictors and outcome
410 variable rather than in model predictions, the BIC seemed more appropriate for our needs
411 [41].

412 The approach of assigning model weights to statistical models functions in short as follows
413 (see [4] for a more extensive discussion of this approach). First, a set of potential models is a
414 priori chosen and for each the model weight is calculated. The models in the set differ by the
415 number and combinations of predictors (explanatory variables) that they include. The model
416 weights reflect the probability of each model being the best-fitting model within the given set
417 of models (model probability, *mPr*) given the data. If one model has a very large probability
418 and all other models have probabilities close to zero there is strong evidence for this single
419 model, i.e. a specific combination of predictors. More often, several models have a
420 considerable model probability. In this case, the current data cannot fully differentiate
421 between the models and they are all potential candidates for explaining an observed pattern
422 (with the given model probabilities). Therefore, it makes sense to consider several models
423 and to discuss what their implications would be.

424 Because we did not have strong a priori notions on how alternative hypotheses were to be
425 reflected in the predictors, we chose the set of potential models as wide as possible. In all
426 our sets the minimal model was the null model consisting of a constant (intercept) only. The

maximum model in all our sets included all potential predictors and all their potential interactions (for the specific cases they are defined below). Where computationally tractable, we conducted an all subset analysis, i.e. all possible combinations of predictors and their interactions were included in the set (function “dredge”, package “MuMIn”; [42]). Where the number of models was computationally intractable, we a priori specified models in addition to the null and the maximum model which consisted of a series of simple models (few predictors and simple interactions) and a series of more complex models which we set up using our prior knowledge. These models were then compared using the package “AICcmodavg” [43] adapted for BIC values.

For the presented models the so called evidence ratio in relation to the null model (E_0) is additionally shown. E_0 indicates how many times more probable the presented models are in comparison to the null model. As with a classical frequentist (p-value-based) approach, predictors are more likely to contribute to a model that has a high probability if the effect of the predictor in question is either consistent though potentially small or strong and potentially more variable. Therefore, we also present the size of the estimated effects and their confidence interval either as numbers or as curves allowing to interpret the biological relevance of the results.

2.6.1 Cognitive bias test

A generalised linear mixed-effects model based on the binomial distribution was used to analyse the go/no-go reaction of the sheep with session number nested in animal identity as the random effect. Fixed effects of the maximum model were housing condition (factor with two levels: predictable rich versus unpredictable poor), the position of the box (continuous as the proportion of the distance from the negative location) and their interaction.

The proportion of sheep that achieved the final learning criterion and the number of sessions needed to achieve this criterion were compared between the two housing groups using

Fisher's exact test and a Mann-Whitney-U-test, respectively. Sheep achieving the learning criterion did so in at most 32 sessions – all other sheep went through at least 34 sessions.

2.6.2 Cortical brain activity

Before statistical evaluation, the [O_2Hb] and [HHb] needed to be further processed because we had the impression that too much smoothing of the signal occurred when we compared signals of individual stimuli to time-triggered medians that we had used before (block-averaging [6,19]). We had to overcome two technical issues in order to evaluate the single individual stimuli. A numerically efficient approach was found with the implementation lmer for mixed-effects models that stores sparse matrices efficiently (package lme4; [40]) allowing to calculate and compare our models using a large number of observations. Also, we accounted for a high one-step temporal auto-correlation in our 1 Hz recordings (see [6,19]) by averaging our data across three seconds. We thus used 5, 15, and 5 values for the pre-stimulus, stimulus and post-stimulus intervals, respectively. We ended up with data on 24 sheep x 3 conditions x up to 12 repetitions x up to 8 light paths x 25 values throughout each stimulus. This resulted in 164'770 rows of data (95% of the potential observations) because some stimuli and paths were excluded because of movement artefacts.

To satisfy statistical model assumptions, [O_2Hb] and [HHb] were transformed for use as outcome variables according to [19]. Random effects were single light paths nested within stimulation number nested within session and in sheep identity. The fixed effects consisted of housing condition (factor with two levels: predictable rich versus unpredictable poor), presumed stimulus valence (ordered factor reflected by a linear and quadratic term for pricking, pressure and kneading coded as 1, 2 and 3, respectively), time course throughout the stimulation (natural spline function of a continuous variable to allow for an unrestricted but smooth signal in time), and location on the head (laterality: indicator for left versus right hemisphere; longitudinal position: indicator for cranial versus caudal location; measurement depth: deep versus superficial measurement). Reflecting stimulus valence as a linear and quadratic term instead of as an ordered factor is equivalent. Using a linear and quadratic

479 term allows the quadratic term to be dropped, though, if changes in response to stimulus
480 valence are close to linear on the transformed scale. The full model included all of these
481 fixed effects, as well as all their potential interactions. Based on this model, degrees of
482 freedom for the spline were selected from among the numbers 5, 9, and 13 in the first step of
483 the evaluation (cf. [19]) influencing the amount of curvature possible in the model estimates.

484 To specify the necessary fixed effects, we set up a total of 33 models, with the simplest
485 model being the null model containing only a constant, and the most complex model being
486 the one with all effects and their potential interactions using the degrees of freedom as
487 selected in the first step (2 models). 16 intermediate models were set up including simple
488 main effects and interaction models focusing on our main predictors (housing condition,
489 stimulus valence, time course) and more complex models explicitly using spatial information
490 in respect to position on the head. For these we used our previous knowledge on the
491 occurrence of longitudinal [6] and lateralised differences in reactions to emotional stimuli [19].
492 An additional 15 models were set up that simplified the ordered factor for valence to a linear
493 relationship on the transformed scale.

494 2.6.3 Behavioural measurements

495 The numbers of aversive behaviour patterns, vocalisations, rearing up the walls, and nibbling
496 shown in each of the session were log-transformed for use in the statistical model. Prior to
497 transformation, 0.5 was added to the original counts to ensure that all values were > 0. The
498 maximum model included the fixed effects of stimulus valence (coded as a linear and
499 quadratic term as described above), housing condition (factor with two levels), and their
500 interaction. Random effect was sheep identity.

501 Movement of the sheep and their ears was log-transformed and all proportions were logit-
502 transformed for use as outcome variables in one mixed-effects model for each outcome.
503 Fixed effects of the maximum model included stimulus valence (again reflected as a linear
504 and quadratic term), housing condition (factor with two levels: predictable rich versus

505 unpredictable poor), and phase (factor with three levels: pre-stimulus, stimulus, post-
506 stimulus) as well as all their potential interactions. The random effect in these models was
507 the stimulation number nested in session nested in animal identity. Additionally, the
508 proportion of available data for each phase was used as a weight in the evaluation such that
509 more complete phases were weighted more strongly.

510

511 **3 Results**

512 *3.1 Cognitive bias test*

513 One of 12 sheep (8%) of the predictable rich housing group failed to achieve the learning
514 criterion for inclusion into cognitive bias testing, as opposed to 5 of 12 (42%) sheep of the
515 unpredictable poor housing group – a difference not reaching statistical significance, though
516 (Fisher-exact test: $p = 0.16$). In addition, sheep from the predictable rich housing group
517 needed fewer training sessions to achieve the learning criterion of the cognitive bias test
518 (17.5 sessions in the median) than sheep from the unpredictable poor housing group (with a
519 median of 30.5; MWU-test: $V = 67.5$, $p = 0.03$).

520 The probability of sheep opening a box mainly increased with relative distance from the
521 negatively reinforced location (model with main effect of position of the box: $mPr = 0.72$,
522 $E_0 > 1436$; Fig. 1, grey lines). There was weaker evidence that sheep from the unpredictable
523 poor housing group were less likely to approach the boxes compared to sheep from the
524 predictable rich housing group (model additionally including the main effect of housing group:
525 $mPr = 0.25$, $E_0 > 506$; Fig. 1, solid black lines). This additional effect of housing condition was
526 indeed quite considerable in that the sheep from the unpredictable poor housing condition
527 were by 0.27 less likely to open the box in the middle compared to the sheep from the
528 predictable rich housing condition (Fig. 1, horizontal dashed lines). All other models reached
529 negligible model probabilities ($mPr < 0.03$, $E_0 < 58$).

530

531 *3.2 Cortical brain activity in response to stimulus valence*

532 For both outcome variables, [O_2Hb] and [HHb], the smallest degrees of freedom for the
533 natural spline of the time course were found to be sufficient (both *mPr* = 1.00).

534 Strong evidence was found that [O_2Hb] varied with the time course of the stimulus and its
535 valence (including their interaction: *mPr* = 0.91, E_0 = 13.91; the second-most probable model
536 being the null model: *mPr* = 0.06). Rather small effects were found in that [O_2Hb] showed an
537 initial decrease in concentration of about half the stimulus duration, a final decrease in
538 concentration of about half the stimulus duration, and a final increase in concentration
539 starting about mid-way through the stimulus duration with the pricking, pressure, and
540 kneading stimulus, respectively (Fig. 2, top).

541 For [HHb], strong evidence was also found that the time course of the stimulus and its
542 valence could well describe the observed changes (including the interaction: *mPr* = 1.00,
543 E_0 >> 10,000). [HHb] showed a clear decrease throughout application of the pricking
544 stimulus, but only marginal changes in its concentration were observed with the pressure and
545 kneading stimulus. The second-most probable model including the additional effect of
546 housing condition and its interactions with time course and stimulus valence had only
547 negligible evidence (*mPr* << 0.001, E_0 >> 10,000; Fig. 2, bottom, black curves). If one wishes
548 to consider the difference between housing groups at all, animals from the unpredictable
549 poor housing condition showed a marginally more extreme decrease in response to the
550 pricking stimulus as well as the kneading stimulus (Fig. 2, bottom, grey curves).

551 *3.3 Behavioural reactions in response to stimulus valence*

552 Sheep exhibited aversive behaviour patterns specifically in response to the pricking stimulus
553 (main-effects model including valence and valence squared: *mPr* = 0.68; E_0 > 1366), with
554 weak evidence that aversive behaviour patterns were exhibited slightly more often by sheep

555 in the unpredictable poor group (model including the additional effect of housing condition:
556 $mPr = 0.12$; $E_0 > 250$; estimated effects [95% confidence interval] for the unpredictable poor
557 group and the pricking, pressure, kneading stimulus: 9.32 [5.87; 14.75], 2.24 [1.23; 3.77],
558 1.94 [1.06; 3.41]; predictable rich group: 7.13 [4.38; 11.65], 1.63 [0.88; 2.78], 1.40 [0.70;
559 2.55]). Weak evidence was found that sheep from the unpredictable poor housing group
560 vocalised slightly more often ($mPr = 0.22$; $E_0 = 0.43$; unpredictable poor, predictable rich:
561 0.25 [0.09; 0.46], 0.04 [-0.08; 0.18]) and reared up the wall slightly more often (with stronger
562 evidence; model including the main effect of housing condition: $mPr = 0.46$; $E_0 = 1.44$; 0.75
563 [0.39; 1.21], 0.10 [-0.07; 0.34]). There was moderate evidence for slightly less nibbling of the
564 accompanying experimenter when the sheep were confronted with the pricking stimulus
565 (model including the main effects of valence and valence squared: $mPr = 0.58$; $E_0 > 11.6$;
566 pricking, pressure, kneading stimulus: 0.29 [-0.03; 0.79], 1.48 [0.76; 2.57], 1.09 [0.52; 2.02]).

567 Sheep showed an increase in general activity in the stimulus phase, particularly in the phase
568 with the pricking stimulus (model including the main effects of valence, phase and their
569 interaction, as well as valence squared, phase and their interaction: $mPr = 0.89$; $E_0 > 1780$),
570 with additional weak evidence that sheep from the unpredictable poor housing group
571 exhibited about the two-fold general activity (model with the additional main effect of housing
572 condition: $mPr = 0.10$; $E_0 > 200$; Fig. 3a). The same qualitative pattern was found for ear
573 movements ($mPr = 0.94$ with $E_0 > 1882$, and $mPr = 0.06$ with $E_0 > 120$, respectively; Fig. 3b).

574 Horizontal ear postures appeared to be more synchronised, in that the proportion of forward
575 and backward ear postures increased during stimulation independent of the type of stimulus
576 (models with main effect phase: $mPr = 0.79$ with $E_0 = 22$ and $mPr = 0.85$ with $E_0 = 40.5$,
577 respectively; Fig. 3c, d). There was weaker evidence that the proportion of forward ear
578 postures decreased by a factor of about 0.5 (additional main effect of valence: $mPr = 0.10$;
579 $E_0 = 2.6$) and the proportion of backward ear postures increased by a factor of about 2
580 (additional main effect of valence squared: $mPr = 0.05$; $E_0 = 2.3$) from the pricking to the
581 pressure and the kneading stimulus (Fig. 3c, d). There was no evidence that the proportion

582 of passive ears was influenced by any of our predictors (null model: *mPr* = 0.93; Fig. 3e).
583 Finally, there was only marginal evidence that the proportion of left-lateralised ears
584 decreased by 0.3 from the pricking to the pressure and to the kneading stimulus (main effect
585 of valence: *mPr* = 0.05; E_0 = 0.06; Fig. 3f).

586

587 **4 Discussion**

588 *4.1 Cognitive bias test*

589 Our testing paradigm was generally successful, insofar as sheep from both housing groups
590 consistently approached the positively reinforced box on one side, and avoided the
591 negatively reinforced box on the other side. Moreover, sheep from the predictable rich
592 housing group showed a clearly graduated reaction to the ambiguous boxes (Fig. 1, bars).
593 The reaction is less clear in the sheep of the unpredictable poor housing group, which may
594 be due in part to the smaller sample size as fewer sheep achieved the learning criterion. This
595 variability in the choice of the sheep from the unpredictable poor housing condition is likely to
596 be responsible for the fact that the considerable difference between the two housing groups
597 can only be supported with rather weak statistical evidence. This was the case in spite of our
598 serious manipulation of the housing conditions, specifically in terms of stimulus richness and
599 predictability of the housing environment. Nevertheless, the difference was compatible with
600 the notion that the sheep from the unpredictable poor housing group had a more negative
601 cognitive bias, indicative of a more negative mood. This effect was found in a main effect of
602 housing condition indicating a general shift of the reaction and no difference in the steepness
603 of the reaction.

604 It remains unclear, however, why our manipulation of the housing conditions – which we
605 consider to be fairly severe – cannot be substantiated more strongly in terms of mood as
606 reflected in the sheep's choices in the cognitive bias test. Such an influence was found when
607 conditions were experimentally worsened similar to our experiment, by e.g. exposing sheep

608 to repeated unpredictable and aversive events [28], subjecting rats to daily negative
609 interventions at random times [30], and removing enrichment materials in experiments with
610 rats [44]. Similar effects were also found when conditions were experimentally improved [45-
611 48]. Other researchers have found evidence that release from a negative situation induces a
612 positive bias (sheep: [49,50], goats: [51]), or that a manipulation produces no effect [52]. The
613 design of our study was intended to chronically worsen housing conditions of one whilst
614 consistently improving conditions for the other group. We therefore subjected the sheep to
615 the conditions for several months, whereas animals in the previous experiments had been
616 subjected to their specific treatments for several weeks at most. Either our sheep largely
617 adapted to and compensated for their housing conditions in terms of their reactions in the
618 cognitive bias test, or the cognitive bias test is not sensitive to mood shifts caused by such
619 long exposures to the given conditions. This latter explanation is plausible, seeing that in
620 some of the experiments changes in the conditions enlarged the effect in contrast to being
621 kept in a steady condition [53-55]. The decreased sensitivity to long-term conditions could be
622 explained in that relatively more short-term events might interfere with the long-term mood
623 induction more easily. We cannot completely rule out the possibility that the lack of
624 differences between housing conditions found in the current study is an effect of the small
625 number of groups used for mood induction (one group per mood), even though the
626 unpredictability can be assumed to have had an independent effect on each of the sheep. In
627 future studies, it may be advisable to increase the number of groups in which different mood
628 is induced, and to investigate in greater depth how cognitive bias develops over time
629 following changes in the environment.

630 The absolute level of cognitive bias is affected not only by mood, but also by the type of
631 stimuli used in the test (Mendl, personal communication). In our case, the negative stimulus
632 appeared to have been quite effective (qualitative observation). If this effect is strong, it could
633 bias sheep from both housing groups so strongly towards the negative that the housing-
634 induced mood difference is no longer visible.

635 Sheep from the two unpredictable poor housing group were much slower in learning the
636 cognitive bias paradigm. The stimuli encountered during the training seemed to pose a much
637 more difficult problem to them compared to the sheep of the predictable rich housing group.
638 Similarly, Wichman et al. [56] found that a lower number of training trials were necessary for
639 hens that later approached the ambiguous stimulus close to the rewarded side faster and
640 Destrez et al. [57] found learning deficits in sheep that showed a negative cognitive
641 judgement bias, but see also [58,59] who found better learning ability in animals from a
642 presumably poorer mood. These effects might be directly related to an abrupt increase in
643 stimulus richness in the testing situation [28], but could also be mediated by mood, or by risk-
644 proneness, which itself may depend on mood (see e.g. [60] for an example with pigeons).
645 Our difference in learning speed might indicate that in an ideal setting housing conditions
646 used to induce mood and the testing paradigm used to assess mood should not differ too
647 much in the type and amount of stimuli encountered by the animals.

648

649 *4.2 Cortical brain activity and behavioural reactions in response to stimulus valence*

650 Sheep most clearly differentiated pricking from pressure and kneading in that they
651 demonstrated more aversive behaviour, less nibbling, more general activity, more ear
652 movements, more forward and fewer backward ear postures, and a stronger decrease in
653 [HHb] during pricking than during pressure and kneading. Usually, the [HHb] change is on a
654 smaller scale, and is less predictable, though often the opposite of the [O_2Hb] change [34]. In
655 the current experiment, however, neither of these aspects seemed to hold true: changes in
656 [O_2Hb] were smaller than those in [HHb], and the pattern of changes in [HHb] seemed more
657 consistent with a clear decrease throughout the duration of the negative stimulus. This
658 coincides with the observation that [HHb] is a more reliable measurement of cortical
659 activation because [O_2Hb] is much more influenced by superficial haemodynamic changes in
660 the skin above the cortex [61]. We therefore assume that the decrease in [HHb] reflects a
661 general activation in the negative situation, such as seen in goats undergoing a frustrating

experience [19]. Similarly, Cunningham et al. [62] found a decrease in prefrontal activation in response to stimuli ranging from negative to positive valence in humans. In addition, Glotzbach et al. [63] observed a prefrontal activation in women viewing fear-inducing as opposed to neutral pictures that did not seem to be an automatic regulation process. Because the frontal cortex does not appear to play a major role in cortical pain networks (rats: [64]), the reaction we observed towards the negative stimulus are unlikely to be a direct effect of pain caused by the stimulus. Given this interpretation of the brain activation and the pattern found in the behaviour such as more aversive behaviour, increased general activity and increased movement of the ears, it seems indeed likely that pricking was perceived as negative by the sheep. Brain oxygenation seemed to have picked up the differences between the stimuli with a higher sensitivity in that the changes in [HHb], showed larger effects substantiated by stronger statistical evidence compared to the behavioural variables.

Differentiation between pressure and kneading was less pronounced in all of our measurements, with only a weak statistical evidence that forward ear postures further decreased and backward ear postures further increased from pressure to kneading, and that, going from pricking to pressure and kneading, there was a general monotonous decrease in the proportion of time that the left ear was positioned further to the front. Nevertheless, the patterns observed were consistent with the idea that the pressure and kneading stimulus were increasingly positive relative to pricking. Though some of the estimated effects were quite considerable (changes by a factor of two), the weaker differentiation between pressure and kneading may indicate either that pressure was perceived as slightly positive, and/or that the automatic physical kneading was not deemed to be as positive by the sheep as being groomed by a human caretaker [17]. Given the very frontal position of the fNIRS sensor it seems unlikely that the observed cortical activation directly reflected sensory processing. Also, if physical stimulation had been the cause of the patterns observed a monotonous change from pricking to kneading to slight pressure would have been expected but was not observed.

689 Our approach was novel in that it attempted to control for all aspects of the stimulus other
690 than its valence by choosing stimuli that were as similar to each other as possible and using
691 the same sensory modality in all the stimuli. Looking at our results in respect to the general
692 activity, this may only have been successful in parts because general activity increased most
693 strongly during pricking but also during kneading possibly indicating that these two stimuli
694 were more arousing than pressure. Indeed, there was hardly any change from the pre-
695 stimulus to the stimulus and the post-stimulus phase for pressure implying that pressure was
696 only marginally perceived at all. Overall, the differences found by us reflect those of our own
697 [4,16,17] and others' [20, 22] previous research investigating a variety of emotional stimuli,
698 indicating that measurements such as ear movement and postures as well as
699 haemodynamic changes in the frontal cortex do actually indicate the valence of a situation
700 more than e.g. its arousal-induction potential. Because we assumed that the sheep would
701 habituate with different speeds to the different stimuli and adjusted the number of habituation
702 sessions accordingly, the number of habituation session was at least partly confounded with
703 valence of the stimuli. If habituation was the main cause of the observed patterns, we would
704 have expected a monotonous change in reactions from kneading to pricking to slight
705 pressure. This was not the case and we therefore conclude that the aspect of valence of the
706 stimuli was much stronger than the aspect of habituation in this experiment.

707 Although the sheep seemed to differentiate the physical stimuli in a manner consistent with
708 their presumed valence, behavioural reactions and cortical brain activity towards these
709 stimuli could not be shown to be modulated by housing condition. The minor general effects
710 of housing condition implied that sheep from the unpredictable poor housing condition in
711 general experienced the test situation as more negative than those from the predictable rich
712 housing condition, showing more signs of both alertness and aversion. This could be a direct
713 reaction to a wealth of stimuli in the testing environment, which was more challenging for the
714 sheep from the unpredictable poor group, but would also be consistent with the notion that
715 there was a slight mood difference in the sheep from the two housing groups (cf. the
716 discussion of the results in the cognitive bias test). Finally, keeping the stimuli as similar to

each other as possible, apart from the valence, might have reduced the range in valence compared to earlier studies, and this narrow range could have made it more difficult to detect an interaction with housing condition. Given the minor effects of these presumed mood differences, little evidence can be gleaned from this study pointing towards either a general tainting effect [2] or towards greater reactivity in individuals in a more negative mood than in those in a more positive mood [4,6] though the weak pattern found in [HHb] would point towards the former in that the reactions to pricking as well as to kneading differ towards a reaction consistent with being more negative.

5 Conclusions

Sheep reacted consistently towards stimuli according to their presumed valence, in that they were increasingly less attentive towards, as judged by their ear postures as movements, and showed fewer signs of aversion in response to increasingly positive stimuli. The absence of a strong effect of a long-term manipulation of the predictability and stimulus richness of the housing environment, however, was possibly due to a lack of sensitivity of the cognitive bias test after long (i.e. several months') exposure of the sheep to the housing conditions in question. Alternatively, the sheep's reaction to physical stimuli might not be changeable by mood in order to avoid the potentially harmful effects of these stimuli.

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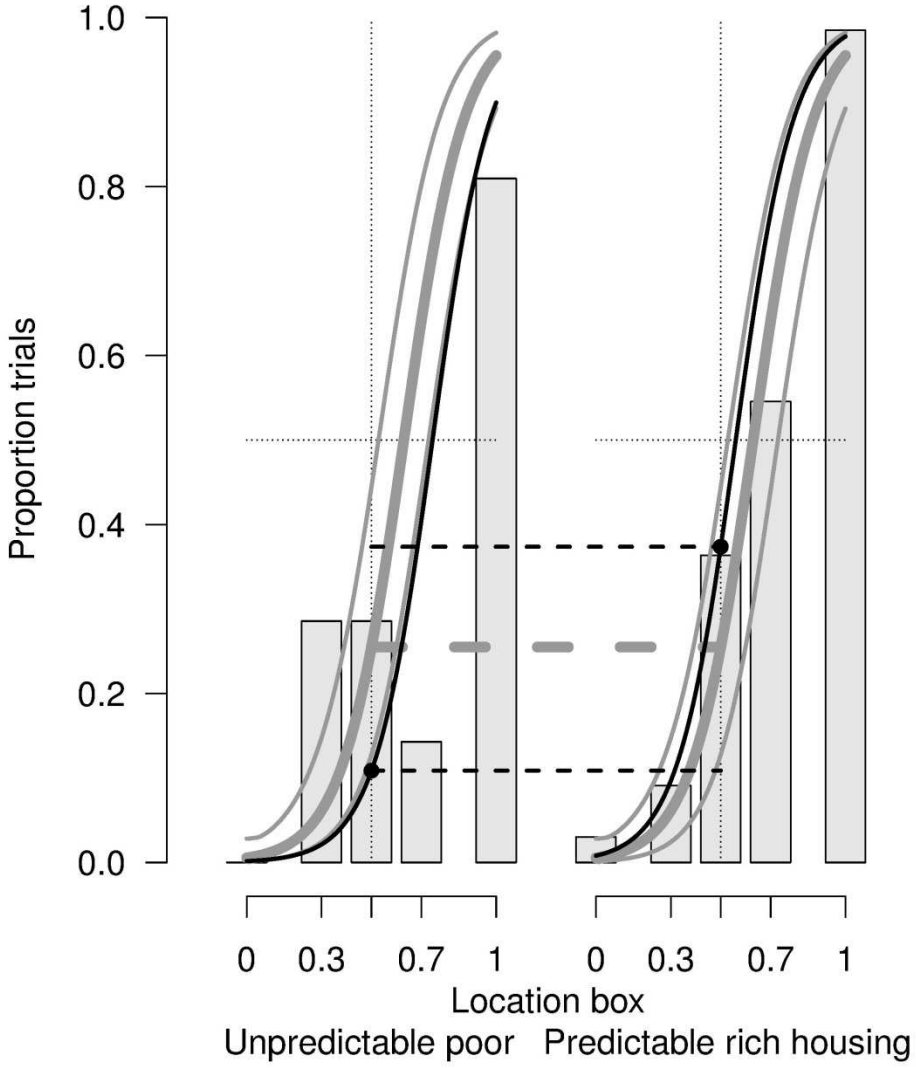
912 **Figure captions**

913 Figure 1: Results from the cognitive bias test. Bars represent the proportion of trials in which
914 sheep (n = 11 in the predictable rich housing group; n = 7 in the unpredictable poor housing
915 group) approached the corresponding boxes at the relative distances from the negatively
916 reinforced box at location 0. Grey lines indicate model estimates and 95% confidence
917 intervals of the best model including box position as a main effect (identical for both housing
918 groups). Black lines indicate model estimates from the model including the main effects of
919 box position and housing condition. Horizontal dashed lines indicate estimated proportions at
920 relative distance 0.5 (grey: main effect box position; black: main effects of box position and
921 housing condition). Dotted lines indicate the proportion 0.5 at relative distance 0.5.

922 Figure 2: Average changes in concentration of O_2Hb (top) and HHb (bottom) throughout the
923 application of three different physical stimuli in sheep from the unpredictable poor and
924 predictable rich housing groups. Black lines: models with highest model probabilities (O_2Hb
925 and HHb: main effects of stimulus valence as a factor and time as well as their interaction).
926 Grey lines: models with second-highest model probability (O_2Hb: null model; HHb: main
927 effects of stimulus valence as a factor, time, and housing condition, plus all possible
928 interactions). Thin lines: 95% confidence intervals. See text for further information on the
929 models. No original data is shown because up to 1152 signals were averaged per curve (12
930 sheep x up to 12 repetitions x up to 8 light paths).

931 Figure 3: General activity (distance covered, m/s, a), ear movements (degrees/s, b),
932 proportion of forward (c), and backward (d) ear postures, proportion of time that ears were
933 passive (e), and proportion of time the left ear was further to the front than the right ear (f) as
934 a function of sheep's housing group (unpredictable poor and predictable rich), stimulus type
935 and phase of the stimulus (Pr = pre-stimulus, S = stimulus, Po = post-stimulus). Boxplots
936 indicate data range as well as median, lower, and upper quartile. Thick black lines: model
937 estimates; thin black lines: 95% confidence intervals. Y-axes are cropped in a-d to enhance
938 visibility of the pattern reflected by the statistical estimates.


1.0
0.8
0.6
0.4
0.2
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Proportion trials
0
0.3
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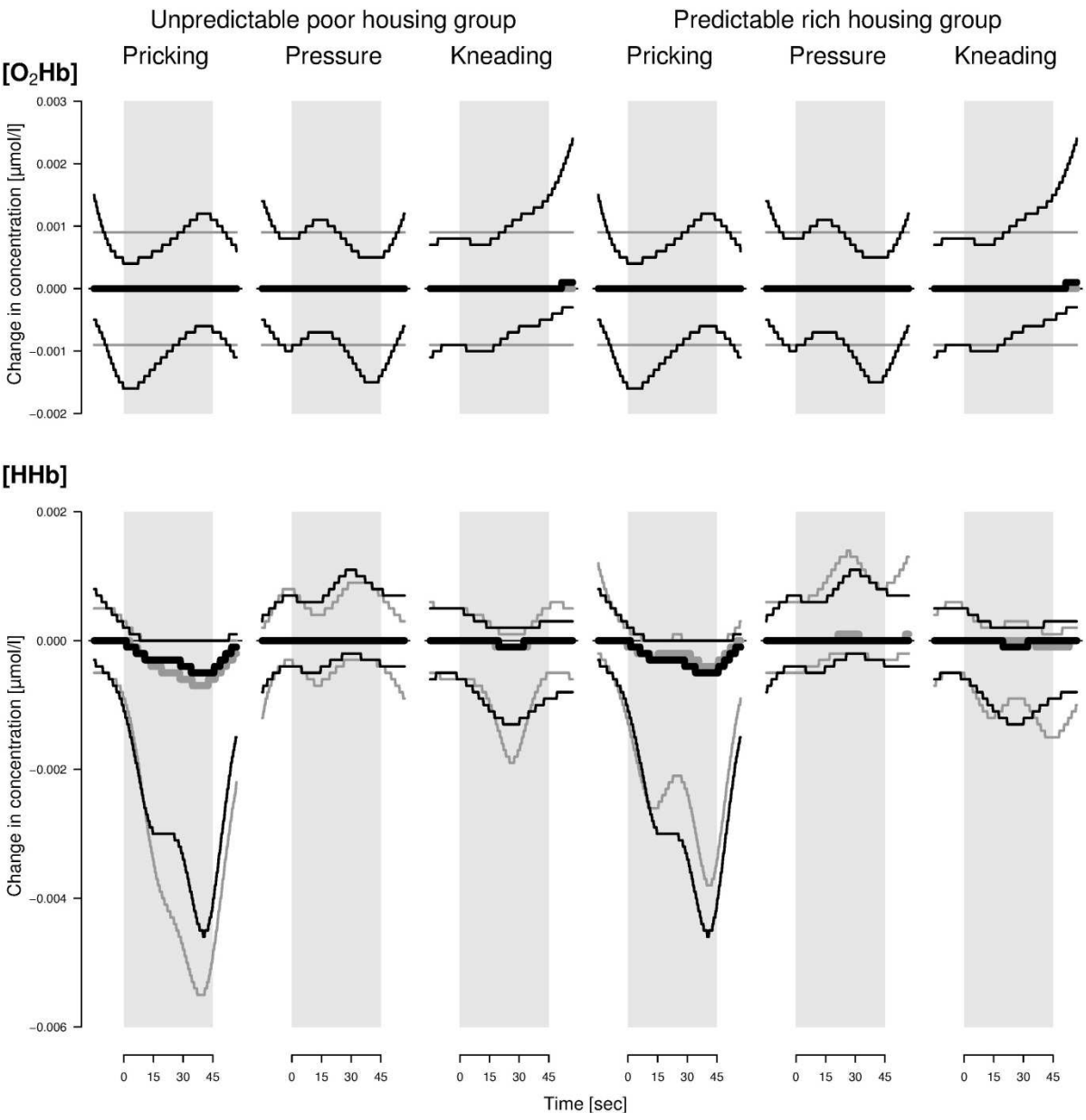
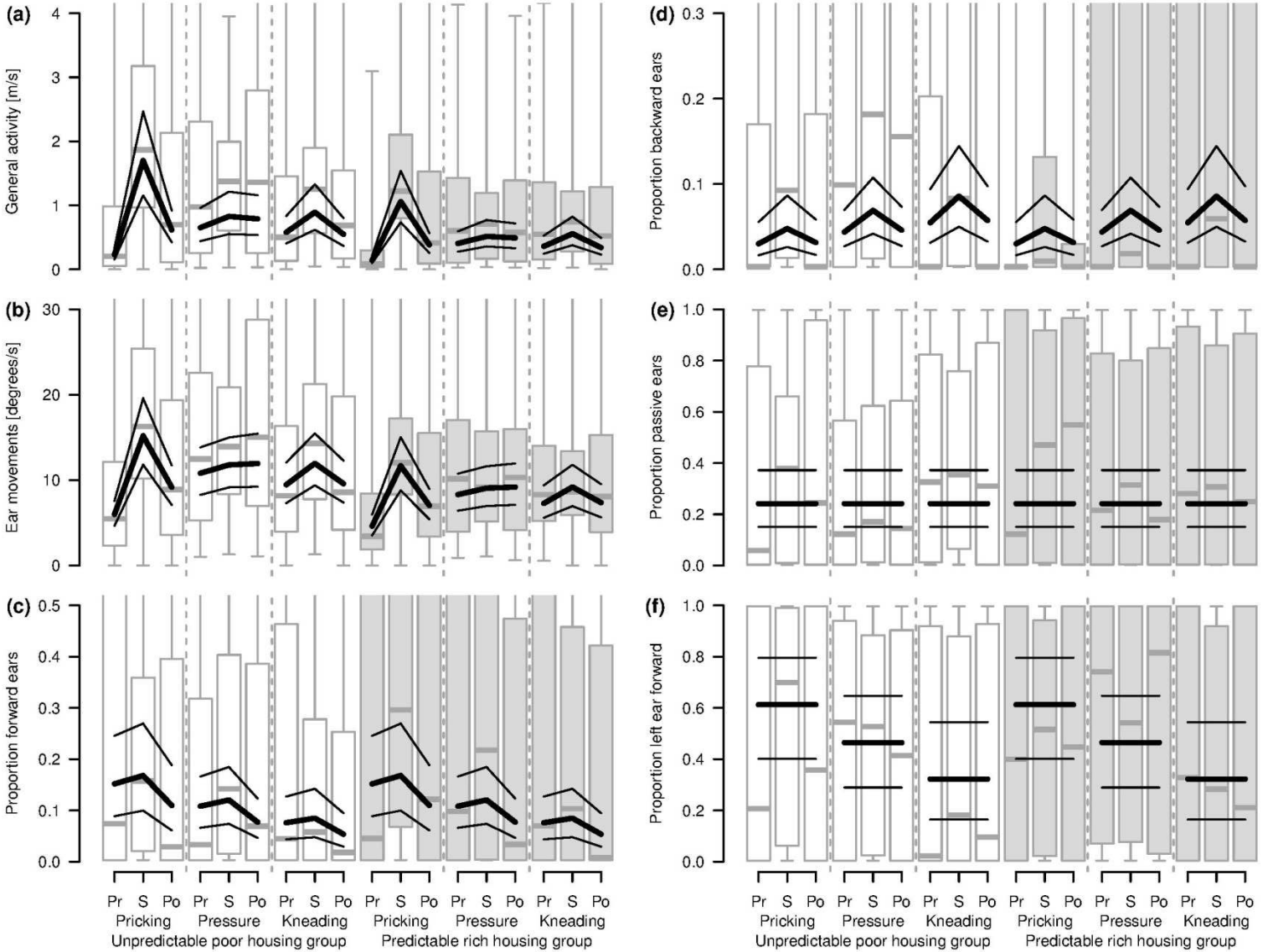
1
0
0.3
0.7
1
Location box
Unpredictable poor
Predictable rich housing


Unpredictable poor housing group
Predictable rich housing group
Pricking
Pressure
Kneading
Pricking
Pressure
Kneading
[O2Hb]
Change in concentration [µmol/l]
0.003
0.002
0.001
0.000
−0.001
−0.002
[HHb]
Change in concentration [µmol/l]
0.002
0.000
−0.002
−0.004
−0.006
0 15 30 45
Time [sec]


(a)
General activity [m/s]
(b)
Ear movements [degrees/s]
(c)
Proportion forward ears
(d)
Proportion backward ears
(e)
Proportion passive ears
(f)
Proportion left ear forward
Pr S Po
Pricking
Pressure
Kneading
Unpredictable poor housing group
Predictable rich housing group